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Sidney Halle
Arthur Brown

FEBRUARY 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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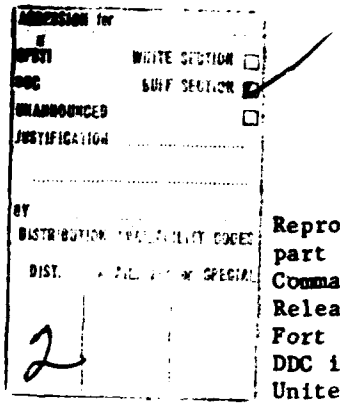

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MODE OF ACTION OF 5-AZACYTIDINE ON ARBOVIRUSES

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BIOLOGICAL SCIENCES LABORATORIES

Project 1B061102B71A

February 1969

ABSTRACT

5-Azacytidine (5-AzaC) was previously shown to be an effective mutagen for arboviruses, particularly the attenuated strain (T) of Venezuelan equine encephalitis (VEE) virus. The drug inhibited viral biosynthesis early in the growth cycle. Its effect was evident and was irreversible (fixed) within the first 5 hours after infection of chick embryo (CE) monolayers. Using eastern equine encephalitis (EEE) virus or Ets-4 (a temperature-sensitive mutant of EEE) in pulse-labeled experiments, 5-AzaC did not show any significant effect on the initiation or rate of total RNA synthesis but did lower the cumulative uptake of C^{14}-uridine in 6-hour infected cells. The lower cumulative uptake may be the result of breakdown of RNA synthesized in the presence of 5-AzaC as hypothesized for bacteria. Sucrose gradient analysis of the different viral RNA species in infected cells revealed a somewhat diminished 20S and 27S RNA fraction in 6-hour infected cultures treated with 25 μg/ml 5-AzaC but the 45S fraction was not affected. The viral RNA synthesized in the presence of 5-AzaC was significantly labile upon phenol extraction, having only 2 to 5% of the infectivity of controls, yet it showed approximately the same quantity of 45S RNA; the latter is normally the most infectious of the known species of viral RNA. The data accumulated on the mutagenic and inhibitory effects of 5-AzaC suggest that it acts directly on viral RNA and support the notion yet to be proved that it is incorporated into the viral RNA.

I. INTRODUCTION*

In a previously published paper,** data were presented to document the mutagenic action of 5-azacytidine (5-AzaC) on several arboviruses. Preliminary evidence was given on the time and possible mode of action of 5-AzaC on viral RNA synthesis. This paper presents evidence confirming and extending those findings.

II. MATERIALS AND METHODS

Our experiments were performed with 24-hour chick embryo (CE) monolayers infected with strains of VEE or EEE virus overlayed with liquid minimal media containing 10 to 25 μg/ml 5-AzaC. Cultures were maintained at 37 C in 5% CO_2 atmosphere and virus yields were assayed as plaques on 24-hour CE monolayers that were stained and scored after 2 days' incubation. Studies on 5-AzaC action within the viral growth cycle were performed with T virus, an attenuated strain of VEE virus that forms small plaques. In experiments designed to examine the effect of 5-AzaC on RNA metabolism and synthesis, we used EEE and Ets-4 virus, a temperature-sensitive strain of EEE described by Zebovitz and Brown.*** Advantage was taken of the increased RNA synthesis described for Ets-4. In these experiments the Zebovitz minimal medium containing uridine-2-C^{14} (5 μc/200 ml), insulin, and actinomycin D was used to overlay infected CE monolayers. At various sampling intervals, supernatant medium was removed for assay of the virus pfu titer, and monolayers were harvested and treated with trichloroacetic acid (TCA) for assay of the C^{14} incorporated into the insoluble product. Counts were made in a Packard liquid scintillation counter.

The earlier studies on the mechanism of 5-AzaC action involved the addition of various nucleic acid precursors (especially uridine) as antagonist to the potent analogue 5-AzaC. Those studies, in which the analogue (5-AzaC) and the antagonist (uridine) were maintained throughout the growth cycle from the times of introduction, showed that the early stages of the viral growth cycle were the most sensitive to the initiation of inhibition and to the reversal of both lethal and mutagenic effects. In recent experiments described below in which medium containing 5-AzaC was replaced with control medium at various times during infection, it was found unnecessary for the drug to be present throughout the complete growth period to affect the final (25-hour) titer.

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** Halle, S. 5-Azacytidine as a mutagen for arboviruses. 1968. J. Virol. 2:1228-1229.

*** Zebovitz, E.; Brown, A. 1968. Pattern of viral RNA synthesis in a temperature-sensitive mutant of eastern equine encephalitis virus. Bacteriol. Proc., p. 163.

III. RESULTS

Table 1 shows that the effect of 10 μg/ml of 5-AzaC during the first 5 hours postinfection could not easily be reversed by washing out and refeeding the culture with fresh control medium. When 5-AzaC addition was delayed 5 hours postinfection, no inhibition of T virus growth was noted in the 25-hour titer. This again illustrates that the early part of the viral cycle is critical for 5-AzaC action. In addition, it indicates that the 5-AzaC effect was "fixed" (became irreversible) early in the viral growth cycle.

TABLE 1. EFFECT OF DURATION OF 5-AzaC TREATMENT ON T VIRUS

Time of 5-AzaC Treatment (10 μg/ml), hours	Time of Medium Replacement, hours	Supernatant Titer at 25 Hours, pfu/ml	Infectivity, %
-	-	7.7×10^7	100
-	3	8.2×10^7	106
-	5	8.2×10^7	106
0 to 3	3	1.2×10^6	1.6
0 to 5	5	8.8×10^5	1.1
3 to 25	-	3.5×10^6	4.5
5 to 25	-	7.5×10^7	98.0
0 to 25	-	8.9×10^5	1.2

In kinetic experiments with T virus, we found that the inhibition of replication due to 5-AzaC was also detectable early (Fig. 1). When 5-AzaC was added at the time of infection it was not necessary to assay at 24 hours to show the effect; the titer observed at 5 hours was already significantly depressed compared with controls, implying a direct rather than indirect mechanism of action. If uridine was added simultaneously at the time of infection, it neutralized the inhibitory effect of 5-AzaC.

Our present data and previously published results led us to examine the effect of 5-AzaC on a molecular level, that is, on viral RNA metabolism and synthesis in radioactive tracer experiments. One of the initial objectives was to determine and compare the rates of viral RNA synthesis of HER and Hts-4 in the presence of 5-AzaC in a pulse-labeling experiment with uridine-C^{14}. The label was introduced 20 minutes prior to sampling time in the course of the virus growth experiments described above.

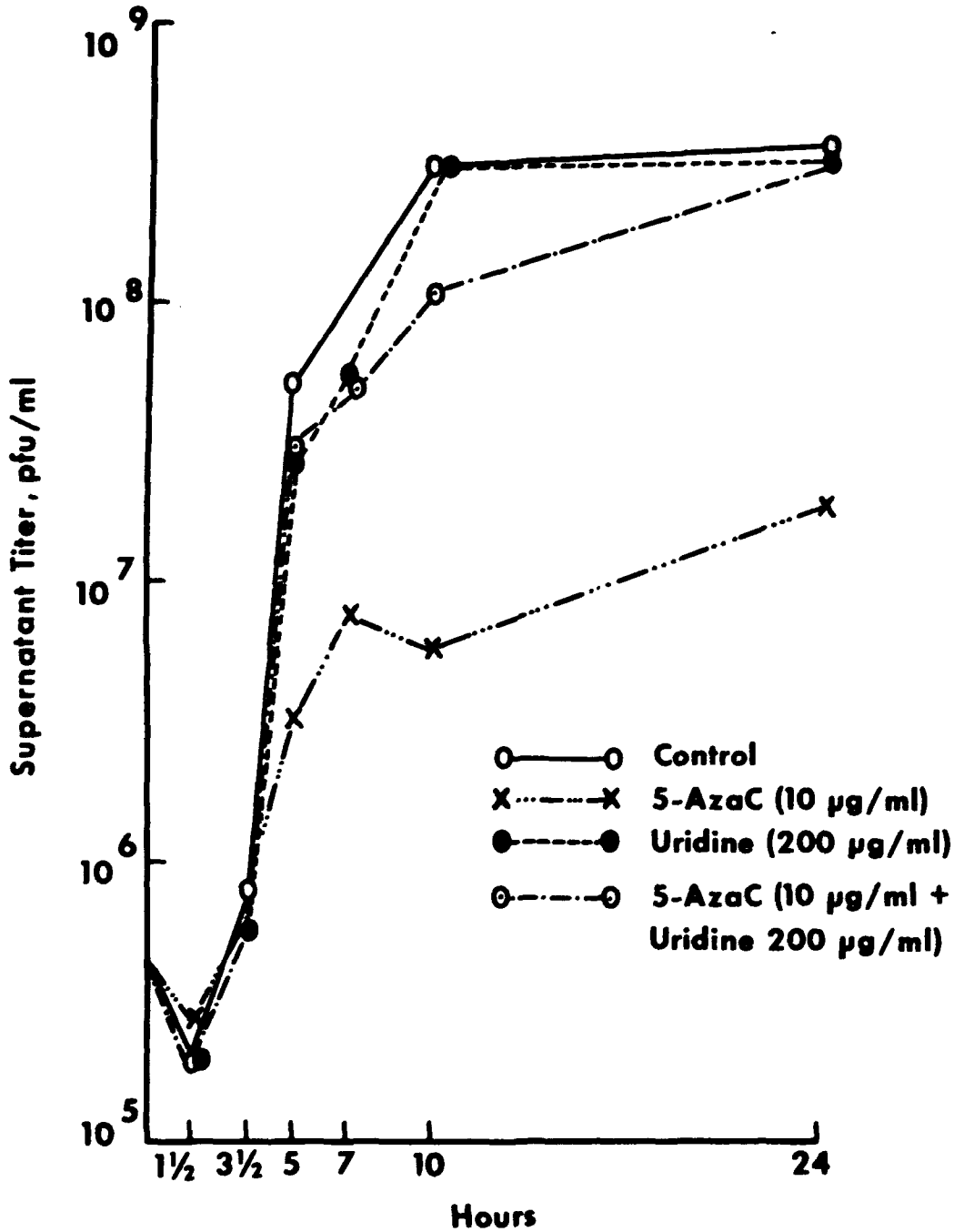

FIGURE 1. T Virus Growth Kinetics in the Presence of 5-AzaC and Uridine.

As seen in Figure 2, the maximum rate of synthesis of EEE viral RNA occurred at approximately 4 hours' postinfection. For Ets-4 the maximum rate occurred at 6 to 7½ hours and sharply declined at 10 hours. 5-AzaC (10 μg/ml) did not significantly affect the time of initiation of synthesis nor the rate of synthesis compared with control cultures. Nevertheless, this level of 5-AzaC (10 μg/ml) had clearly been shown to reduce the infectious virus titer significantly. It was hoped that a clue to the characteristics of the RNA produced might be found in experiments on the effect of 5-AzaC on cumulative or total incorporation of uridine-C^{14}.

Cultures treated with 5-AzaC and then infected with EEE or Ets-4 showed approximately 10% less incorporation of uridine-C^{14} at 6 hours than corresponding untreated control cultures (Table 2), suggesting that the viral RNA, that appears to be made at a normal rate and time, may have subsequently broken down. The loss in virus titer seen in cultures treated with 5-AzaC and the relatively high C^{14} incorporation observed as expected in Ets-4 served as internal controls. Other experiments, in which the concentration of 5-AzaC was varied and in which the patterns of C^{14} uptake and breakdown were followed, showed a maximum accumulation for both EEE and Ets-4 at about 10½ hours' postinfection. However, no marked effect of 5-AzaC was noted. The relatively small effect on cumulative radioactive incorporation did not appear to be sufficient to account for the much larger differences in viral yield between 5-AzaC-treated and control cultures unless a catalytic process could account for the magnified or amplified effect from RNA to infectious virus. If we assume 5-AzaC is incorporated during normal synthesis and causes some breaks in viral RNA, our TCA precipitate method may also precipitate the resulting fragments and it would be difficult to detect quantitative differences in incorporation between 5-AzaC-treated and control cultures.

The next experiment attempted to follow the fate of the radioactive label in conjunction with sucrose gradient centrifugation. RNA extracted from Ets-4-infected CE monolayer cultures were layered on established gradients of sucrose (15 to 35%) and centrifuged at 23,000 rpm for 16 hours on a Spinco 25S rotor. These early experiments showed some indication that 5-AzaC (25 μg/ml) lowered incorporation into 20S and 27S viral RNA but did not affect incorporation into the normally infectious 45S viral RNA.

Table 3 shows that when relative amounts of incorporation into specific RNA were compared, Ets-4-infected cultures incorporated about two times the amount of label into 27S fraction compared with label in the 20S fraction. In 6-hour infected cultures treated with 5-AzaC, the ratio of 27S to 20S RNA was even higher (three times). Overall incorporation into both these lighter fractions was lower relative to 45S, which is a further indication that the RNA species which are detected earliest in infection also are most affected by 5-AzaC in Ets-4 cultures.

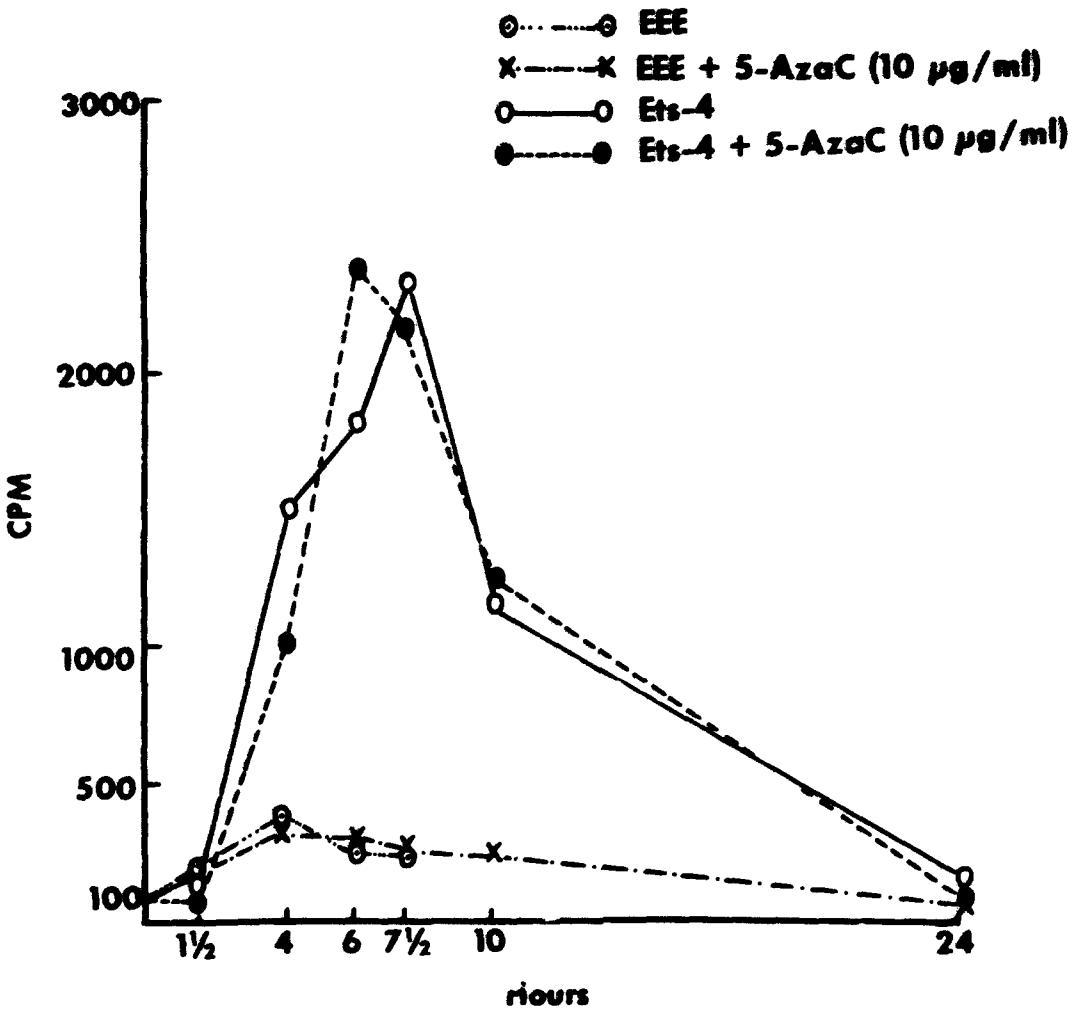

FIGURE 2. Rate of RNA Synthesis (Uridine-^{14}C Incorporation) in EEE and Ets-4 Cultures in Presence and Absence of 5-AzaC (Pulse-Labeling Experiments).

TABLE 2. EFFECT OF 5-AzaC ON URIDINE-C^{14} INCORPORATION IN EEE AND Ets-4 INFECTED CE MONOLAYER CULTURES[a]

Virus	5-AzaC Concn, µg/ml	Supernatant Virus Titer, pfu/ml	C^{14} Incorp. TCA Ppt, counts/min
EEE	0	2.3×10^{10}	7,000
	10	7.6×10^{8}	6,200
Ets-4	0	1×10^{8}	13,200
	10	2×10^{6}	11,900

a. All determinations were made 6 hours after infection.

TABLE 3. RATIO OF INCORPORATION INTO SPECIFIC VIRAL RNA IN Ets-4 INFECTED CE CULTURES

Strain	Time of Sample, hours	5-AzaC Treatment, µg/ml	Ratio of cpm (Uridine-C^{14}) into Specific Fractions (Sucrose Gradient)	
			27S/45S	20S/45S
Ets-4	6	-	5.6	2.9
Ets-4	6	25	3.3	1.1
Ets-4	8	-	5.4	3.3

When these findings were extended through sucrose gradient analysis on sequential samples taken during the course of infection, cultures treated with 5-AzaC compared with controls showed no striking differences in time of appearance or in the species of RNA observed; there was again, however, a smaller overall radioactive incorporation into treated cultures.

As an adjunct to these studies, data were obtained that did show a marked difference in cultures treated with 5-AzaC that may provide a significant positive clue to the nature of 5-AzaC action on (RNA) arboviruses (Table 4). As noted previously, 5-AzaC decreases the titer of both supernatant and intracellular virus. When RNA is extracted from treated and untreated cultures and assayed for infectious RNA (IRNA), an interesting fact is uncovered. When compared with supernatant virus titer (pfu/ml), the infectivity of RNA extracted from 5-AzaC cultures is only 2 to 5% of the infectivity extractable from untreated cultures. This further strongly indicates a lability of viral RNA produced in the presence of 5-AzaC which was only suggested from previous data on cumulative uptake of labeled uridine.

TABLE 4. EFFICIENCY OF IRNA[a] RECOVERY FROM 5-AzaC-TREATED Ets-4 CULTURES

Sample Time, hours	5-AzaC Treatment, 25 μg/ml	Titer, pfu/ml[b] Supernatant Virus	IRNA	Relative IRNA Recovery, IRNA/Virus	Relative Efficiency of IRNA-5-AzaC/IRNA Control, %
0 to 4	-	2.7×10^8	7×10^7	0.26	
0 to 4	+	4.7×10^7	6×10^5	0.013	5
0 to 8	-	4.2×10^8	4×10^7	0.095	
0 to 8	+	1.9×10^6	3×10^3	0.0016	1.7

a. Infectious RNA.
b. Each sample was the pooled content of five CE monolayer cultures; total supernatant was 10 ml, total IRNA extract was suspended in 2 ml.

The clear-cut experimental indicator of this lability thus far obtained has been reflected in lowered infectivity in both the yield of whole virus, in the IRNA, and in mutagenesis reported previously. There are other approaches to demonstrate lability of the RNA that we have not yet tested. Perhaps the concentration of 5-AzaC in our present methods is too low, or the Ets-4 strain with its high RNA activity masks an effect which we cannot observe. In addition to these tests other biochemical and biophysical approaches are being initiated to test whether 5-AzaC is incorporated into the RNA, whether the RNA produced is labile, and whether the RNA is biologically active in spite of an association with a decreased infectivity.

IV. SUMMARY

Data have been presented relative to the time and mode of action of 5-AzaC on viral RNA synthesis. In experiments with the attenuated T mutant strain of VEE virus it was confirmed that the early part of the viral growth cycle is critical for 5-AzaC action. Further, the inhibition was evident during the first 5 hours of infection where it also became irreversible. The initiation and rate of viral RNA synthesis of EEE and Ets-4 cultures treated with 5-AzaC did not significantly differ from untreated control cultures. However, the total (cumulative) RNA produced (measured as uridine-C^{14} incorporation) was somewhat less in cultures treated with 5-AzaC. There was also some indication that a relatively small amount of 20S and 27S RNA was found in treated cultures compared with control Ets-4 infected cultures, but the quantities of the normally infectious 45S RNA were the same. Besides the inhibitory effect of 5-AzaC on virus yield, an important new observation was made that the viral RNA made in the presence of 5-AzaC was highly labile in terms of infectivity when compared with untreated controls, although the quantities of the normally infectious 45S species of RNA were alike. Data obtained thus far strongly suggest a direct 5-AzaC effect on viral RNA that is subtle and not easily observed by the biophysical and biochemical methods used to date, but is clearly demonstrable by the sensitive biological attributes of mutagenesis and infectivity.

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13. ABSTRACT

5-Azacytidine (5-AzaC) was previously shown to be an effective mutagen for arboviruses, particularly the attenuated strain (T) of Venezuelan equine encephalitis (VEE) virus. The drug inhibited viral biosynthesis early in the growth cycle. Its effect was evident and was irreversible (fixed) within the first 5 hours after infection of chick embryo (CE) monolayers. Using eastern equine encephalitis (EEE) virus or Ets-4 (a temperature-sensitive mutant of EEE) in pulse-labeled experiments, 5-AzaC did not show any significant effect on the initiation or rate of total RNA synthesis but did lower the cumulative uptake of C^{14}-uridine in 6-hour infected cells. The lower cumulative uptake may be the result of breakdown of RNA synthesized in the presence of 5-AzaC as hypothesized for bacteria. Sucrose gradient analysis of the different viral RNA species in infected cells revealed a somewhat diminished 20S and 27S RNA fraction in 6-hour infected cultures treated with 25 µg/ml 5-AzaC but the 45S fraction was not affected. The viral RNA synthesized in the presence of 5-AzaC was significantly labile upon phenol extraction, having only 2 to 5% of the infectivity of controls, yet it showed approximately the same quantity of 45S RNA; the latter is normally the most infectious of the known species of viral RNA. The data accumulated on the mutagenic and inhibitory effects of 5-AzaC suggest that it acts directly on viral RNA and support the notion yet to be proved that it is incorporated into the viral RNA. ( )

14. Key Words

*Mutagens
*Arboviruses
*Venezuelan equine encephalomyelitis virus
*Eastern equine encephalomyelitis virus
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